Synexin Enhances the Aggregation Rate but Not the Fusion Rate of Liposomes†

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ABSTRACT: The effect of synexin on the calcium-induced fusion of large unilamellar liposomes was studied by using two assays for the mixing of aqueous contents. The results were analyzed in terms of the mass action kinetic model, which describes the overall fusion reaction as a two-step sequence consisting of a second-order process of liposome aggregation followed by a first-order fusion reaction. By using several different lipid compositions and varying the electrolyte composition, it was possible to select the rate-limiting step of the overall fusion process. When aggregation was the rate-limiting step, as in the case of Ca^{2+}-induced fusion of phosphatidylserine (PS), phosphatidate (PA)/phosphatidylethanolamine (PE) (1:3), and PS/PE (1:3) liposomes, synexin increased the overall fusion kinetics by increasing the aggregation rate constant (up to 100-fold). When aggregation was rapid compared to destabilization of apposed membranes, i.e., fusion was rate limiting, synexin either had no effect or reduced the overall fusion kinetics. In one such case involving liposomes composed of PA/PS/PE/phosphatidylcholine (PC) (10:15:65:10), synexin reduced the fusion rate constant by 50%. The effect of calcium-induced synexin polymerization was investigated by preincubation of synexin with calcium prior to addition of liposomes. Prepolymerization by Ca^{2+} always decreased the activity of synexin such that it was less than the activity of an equal amount of untreated monomers. However, it was found that the activity of synexin monomers polymerized to an average hexameric size was greater than that of one-sixth as many untreated monomers, with respect to the liposome aggregation rate constant. Neither polymers nor monomers increased the fusion rate constant.

Synexin is a calcium-activated protein, originally isolated from bovine adrenal medulla (Creutz et al., 1978), but found in many tissues (Creutz et al., 1980; Morris et al., 1982; Meers et al., 1987a). The bovine protein can cause Ca^{2+}-dependent aggregation of isolated chromaffin granules (Creutz et al., 1978, 1983), specific granules from human neutrophils (Meers et al., 1987a) and other membranes (Morris & Hughes, 1979). Because of this activity, it is considered to be a cytoplasmic Ca^{2+} receptor to promote close apposition of chromaffin granules to the plasma membrane and fusion of the membranes in the process of secretion of epinephrine (Creutz et al., 1979). While granule aggregation was demonstrated at Ca^{2+} concentrations greater than 6 μM (Creutz et al., 1978), binding of synexin to chromaffin granules has been observed at as little as 4 μM Ca^{2+} (Creutz & Sterner, 1983). This binding appears to involve phospholipids and not proteins in the granule membranes (Morris et al., 1982). Synexin also undergoes Ca^{2+}-dependent polymerization in the absence of membranes (Creutz et al., 1979). It is not yet known whether this polymerization may play a role in the activity of synexin.

Aggregation and fusion of vesicles as mediated by synexin have been monitored by microscopic observation (Creutz et al., 1978), turbidity changes (Ohki & Leonards, 1982), and fluorescent fusion assays (Hong et al., 1981, 1982a,b). By microscopic observation, chromaffin granules aggregated by synexin and Ca^{2+} appear to fuse upon addition of certain free fatty acids (Creutz et al., 1978). Reversibility of turbidity changes by ethylenediaminetetraacetic acid (EDTA)[1] was used to suggest that synexin does not affect the final extent of Ca^{2+}-induced fusion of phosphatidylserine liposomes (Ohki & Leonards, 1982), though irreversible aggregation could play a role in such experiments. The actual rate of the overall fusion process has been measured by using assays which monitor the mixing of the aqueous contents of liposomes. Hong et al. (1982a,b) found that synexin lowers the threshold concentration of Ca^{2+} needed to cause fusion of liposomes of several lipid compositions. This activity of synexin is not found in most other Ca^{2+} binding proteins, such as calmodulin (Hong et al., 1982a). Synexin increases the rate of Ca^{2+}-induced fusion of phosphatidylserine (PS)- and phosphatidate (PA)-containing liposomes. A particularly large effect is observed with PA/phosphatidylethanolamine (PE) liposomes in the presence of 1.5 mM Mg^{2+}, where synexin lowers the amount of Ca^{2+} required for fusion from 0.5–1.0 mM to 10–30 μM. As we describe below, we interpret this as synexin-induced aggregation, due to Ca^{2+} activation, followed by Mg^{2+}-induced fusion. This separation of aggregation and fusion is critical for understanding the possible biological role of synexin.

The fusion kinetics of liposomes have been analyzed previously by a mass action model with two fusion steps: liposome

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[1] Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPA, dipicolinic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; DPX, *p*-xylylenebis(pyridinium bromide); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetate; NTA, nitrilotriacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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aggregation and actual membrane fusion (Nir et al., 1980; Bentz et al., 1983a). In order to better understand the mechanism of action of synexin with liposomes, we have examined the role of synexin in these two steps of membrane fusion. By testing the effect of synexin under conditions where either aggregation or fusion was rate-limiting, we have been able to determine the action of synexin at each step. Using the mass action model for overall fusion kinetics, we have been able to quantitate the effect of this protein on the rate constants for the aggregation and fusion reactions. To help further elucidate the mechanism of the Ca^{2+}-dependent action of synexin in liposome fusion, the role of synexin polymerization was also investigated. An abstract discussing part of this work has been previously presented (Hong et al., 1983a).

Materials and Methods

Bovine brain phosphatidylserine (PS), phosphatidate (PA) (derived from egg phosphatidylcholine), egg phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (transesterified from egg phosphatidylcholine) were purchased from Avanti Polar Lipids (Birmingham, AL). *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), dipicolinic acid (DPA) (99%), and nitrilotriacetic acid (99%) were obtianed from Sigma (St. Louis, MO). $CaCl_2$ (>99%), $MgCl_2$ (>99%), and NaCl (>99%) were from Fisher (Pittsburgh, PA). 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were from Molecular Probes (Eugene, OR). $TbCl_3 \cdot 6H_2O$ (99.9%) was obtained from Alfa (Danvers, MA). Polycarbonate filters were from Nucleopore (Pleasanton, CA). Sephadex G-75 and CNBr-activated Sepharose 4B were from Pharmacia (Piscataway, NJ). Ultrogel AcA 34 was from LKB Instruments (Rockville, MD).

The purity of phospholipids was assessed by thin-layer chromatography on silica gel H plates (Analtech, Newark, DE) in two dimensions using the solvents chloroform/methanol/NH_4OH (65:25:5) and chloroform/acetone/methanol/acetic acid/H_2O (30:40:10:10:5). The purity of all phospholipids could be estimated as 99% or greater.

Purification of Synexin. Bovine liver synexin was purified by using modifications of previously published procedures (Creutz et al., 1978, 1982; Morris et al., 1982). The purification involved three major steps. (1) Bovine liver was homogenized in toluenesulfonyl fluoride containing buffer (600 cm^3 of liver in 1 L of buffer) and cleared of debris by low- and high-speed centrifugations (Creutz et al., 1978). (2) The remaining supernatants were precipitated twice by 22% saturated $(NH_4)_2SO_4$ and resuspended twice. (3) Synexin was then further purified from this material by a column containing covalently bound liposomes as Ca^{2+}- dependent affinity ligands. EGTA was used to elute synexin from the affinity column.

The $(NH_4)_2SO_4$ pellet was resuspended in 150 mM sucrose, 50 mM MES, and 2.5 mM EGTA, pH 6.0, and cleared of insoluble material by centrifugation at 100000*g* for 1 h at 4 °C. Just before the sample was loaded (protein from about one-third of the total preparation) onto the affinity column, 3.5 mM Ca^{2+} and the appropriate amount of NaCl were added to the sample in order to approximately match the osmolarity of the column buffer and to assure that there was some free Ca^{2+} for synexin binding in excess of that Ca^{2+} bound to the 2.5 mM EGTA in the MES buffer. This material was applied to a 15-mL (5 g) affinity column of Sepharose 4B covalently linked to small unilamellar vesicles composed of PS/PC/PE (40:40:20) equilibrated at 4 °C (see below for method of conjugation). The column was eluted with 300 mM NaCl, 100 mM TES, and 1 mM Ca^{2+}, pH 7.4, until all the protein

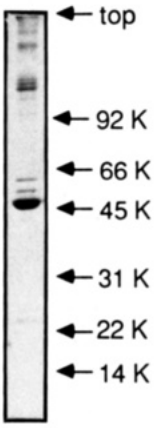

FIGURE 1: Polyacrylamide gel electrophoresis of purified synexin. Approximately 35 μg of synexin was applied to the lane shown. Electrophoresis was performed in sodium dodecyl sulfate as described by Laemmli (1970), and the gel was stained with Coomasie Blue. The gel was poured as a linear gradient of acrylamide from 7% to 17%. The approximate positions of molecular weight standards on the same gel are shown by arrows.

detectable by 280-nm absorbance was eluted. Then, the elution buffer was changed to 300 mM NaCl, 100 mM TES, and 2.5 mM EGTA, pH 7.4, to elute the synexin. Most of the sample activity, as measured by PS liposome aggregation or the fusion assay with PA/PE liposomes, was eluted by the EGTA-containing buffer within a few column volumes. While most of the protein detected by 280-nm absorbance was in the Ca^{2+}-eluted fraction, no activity was detected in these fractions. The affinity column nonspecifically and irreversibly bound a large fraction of the protein applied on its first use, but subsequent runs yielded most of the applied protein. This procedure yielded synexin of estimated 90% purity as shown in Figure 1. The non-synexin protein was spread among a large number of bands on the gel. The small band in the vicinity of 56K may be synexin II (Odenwald & Morris, 1983), though we have not observed its reported activity.

Typical specific activity for purified synexin in terms of liposome fusion is shown in Figure 6. In order to minimize any variations in specific activity among different preparations, synexin from a single preparation was used for any sets of experiments which were compared. Variation in specific activity was mainly due to the existence of inactive aggregates of synexin in some preparations. These aggregates were removed by centrifugation at 100000*g* for 1 h for the experiments shown in Figure 9.

Affinity Column. Vesicles composed of PS/PC/PE (40:40:20) were produced by drying chloroform solutions of these lipids onto the inside of 13 × 100 mm Kimax test tubes, suspending in 0.1 M $NaHCO_3$ and 0.5 M NaCl at pH 8, and then sonicating for three 10-min periods at 22 °C under argon in a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY). An excess of vesicles was added to cyanogen bromide activated Sepharose 4B and incubated in the pH 8 bicarbonate buffer for 1 h at room temperature. Of 49 μmol of phospholipid added, 36 μmol was covalently coupled to 15 mL (5 g) of the column support. Excess reactive sites were then blocked with Tris followed by three washes of 0.5 M NaCl, 0.1 M acetic acid (pH 5.0) alternating with 0.5 M NaCl, and 0.1 M Tris (pH 8.0). The column was finally equilibrated in degassed buffer of 0.3 M NaCl/0.1 M TES, pH 7.4 at 4 °C. The column was washed extensively with 0.3 M NaCl, 0.1 M TES, and 2.5 mM EGTA, pH 7.4, followed by 0.3 M NaCl, 0.1 M TES, and 1 mM $CaCl_2$, pH 7.4, before use. Storage was at 4 °C in the presence of 0.01% sodium azide and the

degassed EGTA-containing buffer. This affinity material appeared to maintain its activity for at least 2 months under these conditions.

Liposome Preparation. Large unilamellar vesicles (liposomes) encapsulating the desired material were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) as modified by Wilschut et al. (1980). For the Tb/DPA contents mixing assay, liposomes encapsulated either (a) 2.5 mM $TbCl_3$ and 40 mM sodium nitrilotriacetate (NaNTA), (b) 50 mM DPA and 20 mM NaCl, or (c) 1.25 mM $TbCl_3$, 20 mM NTA, 25 mM DPA, and 10 mM NaCl. Each encapsulated solution also contained 5 mM TES, and the pH was adjusted to 7.4 prior to encapsulation. For the ANTS/DPX contents mixing assay, liposomes encapsulated either (a) 25 mM 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), 40 mM NaCl, and 10 mM TES at pH 7.4, (b) 90 mM *p*-xylylenebis(pyridinium bromide) (DPX) and 10 mM TES, pH 7.4, or (c) 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 10 mM TES at pH 7.4 (Ellens et al., 1985).

To obtain a relatively uniform size, the liposomes were extruded through polycarbonate filters with a pore size of 0.2 μm followed by 0.1 μm (Szoka et al., 1980). The preparation was centrifuged for 15 min at 10000*g* to remove any remaining very large liposomes. Liposomes were separated from nonencapsulated material by using a 1 × 20 cm column of Sephadex G-75 for each 10 μmol of lipid. For the Tb/DPA liposomes, elution was with 100 mM NaCl, 5 mM TES, pH 7.4, and 1 mM EDTA. EDTA was later adjusted to a final concentration of 0.1 mM. The ANTS/DPX liposomes were eluted from G-75 by 100 mM NaCl, 5 mM TES, pH 7.4, and 0.1 mM EDTA (NaCl buffer). These liposomes were diluted into more NaCl buffer for fusion assays.

The phospholipid concentrations were determined by using a phosphate assay of Bartlett (1959) as modified by Morrison (1964).

Fusion and Aggregation Assays. Two contents mixing assays for fusion were used. The Tb/DPA assay is based on the generation of a highly fluorescent chelation complex of Tb^{3+} and DPA upon mixing of the contents of the liposomes when they fuse (Wilschut et al., 1980). The assay mixture contains a 1:1 ratio of Tb^{3+}- and DPA-containing liposomes in 1-mL final volume of 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA, pH 7.4 (NaCl buffer). In most cases, a small amount (usually 10–20 μL) of a concentrated solution of $CaCl_2$ or $CaCl_2$ with $MgCl_2$ (containing NaCl buffer adjusted to pH 7.4) was injected to initiate the fusion. The 100% fusion level was set by lysing the appropriate amount of Tb^{3+}-containing liposomes with 0.5% sodium cholate in medium containing no EDTA and 40 μM DPA. Any mixing of the Tb^{3+} or DPA contents of the liposomes outside of the liposomes due to leakage during the fusion assay does not result in the formation of Tb/DPA complexes because of the preferential binding of Tb^{3+} to EDTA and, when Ca^{2+} is present, the preferential binding of Ca^{2+} to DPA (because Ca^{2+} is in vast excess to Tb^{3+}). Leakage was monitored by the dissociation of Tb^{3+} from DPA using liposomes containing both (type c above) under conditions identical with the fusion experiments. Leakage of the preencapsulated Tb^{3+}/DPA complex into the medium or influx of the medium into the fusing liposomes results in dissociation of the complex and a loss of fluorescence (Bentz et al., 1983a, 1985). This assay is quite sensitive to both types of leakage (Bentz & Düzgüneş, 1985).

The ANTS/DPX assay for mixing of aqueous contents is based on the collisional quenching of 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) by *p*-xylylenebis(pyridinium bromide) (DPX) upon mixing of contents when liposomes fuse (Ellens et al., 1985). A 1:1 ratio of liposomes encapsulating ANTS and liposomes encapsulating DPX was used for fusion assays. Any mixing of the ANTS or DPX contents of the liposomes outside of the liposomes due to leakage during the fusion assays does not result in the quenching of ANTS because dilution of the DPX decreases quenching to almost zero. Liposomes with coencapsulated ANTS and DPX were used to set 0% fluorescence as well as to measure leakage. Leakage was measured by the increase in fluorescence due to relief of quenching upon dilution of the coencapsulated material into the medium.

The fluorescence was measured on an SLM 4000 fluorometer. The Tb/DPA and ANTS/DPX reaction mixtures were excited at 276 and 360 nm, respectively, and the fluorescence was measured at >530 nm through a Corning 3-68 cutoff filter. All experiments were performed at 25 °C. The contribution of light scattering at the emission wavelength was negligible in both fusion assays.

Fluorescence and 90° light scattering were monitored simultaneously by using the "T-format" of the fluorometer. For the polymerization of synexin, the buffer was set as zero, and the initial scattering from a synexin solution was set at an appropriate value. The change in scattering upon addition of Ca^{2+} was then monitored before addition of liposomes. The fluorometer was precalibrated for the fluorescence of the added liposomes.

Analysis. We model the overall fusion as a mass action process following the initial reaction (see eq 1 under Results) where two monomer vesicles (V_1) aggregate to a dimer (V_2) and then fuse into a doublet (F_2). Higher order reactions will occur, but we monitor only the early events where the relative amount of higher order aggregation/fusion products is small. We have already shown that integration of these kinetic equations yields predicted curves for the Tb/DPA fluorescence which depend upon the primary rate constants (C_{11}, D_{11}, and f_{11}) and the lipid concentration (Nir et al., 1980, 1983; Bentz et al., 1983a,b, 1985; Düzgüneş & Bentz, 1988).

RESULTS

Comparison of Synexin Effects on Aggregation vs Fusion. In the following experiments, the initial rates of fusion of liposomes were measured, and the effects of synexin on these rates were tested. These initial rates are simply the initial slopes of the fusion curves and, as such, represent overall rates which combine both the aggregation rate and the fusion rate, per se (Bentz et al., 1983a,b, 1985). The overall fusion reaction up to the dimer stage is

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2 \qquad (1)$$

where V_1 and V_2 denote the liposome and the aggregated dimer, respectively, and F_2 denotes the fused doublet. Note that we refer to the rate of the second step in this process as the fusion rate while the rate of the whole process is called the *overall* fusion rate. The overall rate of fusion is obviously controlled by whichever step is rate limiting. For example, when the aggregation is very slow compared to the fusion, the aggregation rate controls the overall rate.

Preliminary analysis of the fusion data showed that eq 1 contained the rate-limiting steps of the overall fusion reaction. For example, the reaction of synexin binding to the liposomes certainly occurs, but this step must be fast compared with the liposome aggregation rates after synexin binding, at least 50 times faster (Bentz & Ellens, 1988). Otherwise, our analysis would have shown the effect of this step on the overall fusion

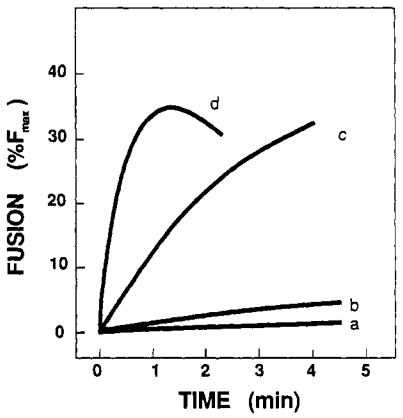

FIGURE 2: Ca^{2+}-induced fusion of 10 μM PA/PE (1:3) liposomes as modulated by synexin. 1 mM Ca^{2+} was added to 1 mL of the liposome solution at time zero to initiate fusion. The cuvette contained (a) no synexin, (b) 1.9 μg of synexin, (c) 4.0 μg of synexin, or (d) 7.5 μg of synexin along with 10 μM phospholipid prior to Ca^{2+} addition. Fusion was measured by the Tb/DPA assay as described in the text. Experiments were performed at 25 °C in NaCl buffer.

kinetics. Likewise, we only fit the data out to times where we are observing just the initial reactions given by eq 1. At these times, the higher order aggregation and fusion reactions do not contribute significantly to the observed fluorescence intensities.

Figure 2 shows typical time courses of fusion, demonstrating the effect of different synexin concentrations on the Ca^{2+}-induced fusion of PA/PE (1:3) liposomes, using the Tb/DPA assay for mixing of aqueous contents. Without synexin, there is essentially no observable fusion within this time frame. Adding 7.5 μg/mL synexin dramatically increases the overall rate of fusion. The enhancement of the overall fusion rate by synexin was also observed when the liposomes were composed of PS/PE (data not shown). In the absence of synexin, the lipid concentration dependence is nearly second order in this system, as would be expected for aggregation rate-limiting kinetics. Therefore, only the effect of synexin on the aggregation step of the overall fusion process is observed.

In order to determine the effects of synexin on both steps of the fusion process, we designed systems in which the change of one parameter could set either the aggregation or the fusion step as rate limiting. First, the ionic composition of the fusion-inducing medium was manipulated (Bentz et al., 1983b; Bentz & Düzgüneş, 1985). In the absence of synexin, the fusion of PS liposomes induced by 3 mM Ca^{2+} was largely aggregation rate limited with a kinetic order of 1.6 in lipid concentration, while the fusion of PS liposomes induced by 4 mM Ca^{2+} and 5 mM Mg^{2+} was largely rate limited by the fusion step per se, with a kinetic order of 1.3 in lipid concentration. Thus, in the first case, an enhanced overall rate of fusion observed in the presence of synexin would probably be due to an effect on the aggregation rate. In the second case, increasing the aggregation rate would not be expected to significantly increase the overall rate of fusion. This is exactly what was seen. Figure 3 shows the initial rate of ANTS/DPX fluorescence as a function of synexin concentration for pure PS liposomes. The open squares show the data when fusion was initiated by addition of 3 mM Ca^{2+}, and the closed squares show the data when fusion was initiated by addition of a mixture of 4 mM Ca^{2+} and 5 mM Mg^{2+}. Similar results were obtained with the Tb/DPA assay (data not shown). Note that while synexin increased the rate of aggregation, it did not inhibit the fusion rate, at least up to the concentrations tested. The difference observed with and without Mg^{2+} is not due to a change in the amount of synexin bound, since gel electrophoresis of supernatants and pellets of multilamellar liposomes composed of PS showed that most of the synexin was bound to the liposomes under both ionic conditions.

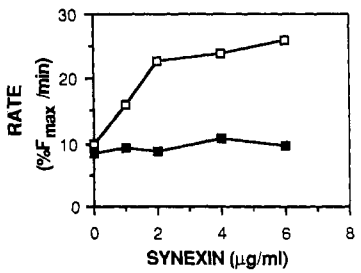

FIGURE 3: Overall rate of fusion of PS liposomes as initiated by 3 mM Ca^{2+} (□) or 4 mM Ca^{2+} and 5 mM Mg^{2+} (■). Ca^{2+} or Ca^{2+} with Mg^{2+} was added at time zero to initiate fusion of a 1-mL solution containing 10 μM PS and the indicated amount of synexin, and the initial rate was measured. The fusion was measured by the ANTS/DPX assay as described in the text. The fluorescence of the fusion curves was corrected for leakage as described in Bentz et al. (1983a) by setting the fluorescence intensity equal to the original intensity plus half of the intensity of the leakage curve at the same time. All experiments were performed at 25 °C in NaCl buffer.

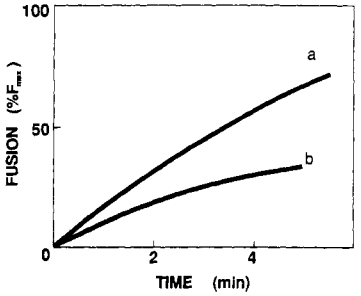

FIGURE 4: Fusion of PA/PS/PE/PC (10:15:65:10) liposomes by 5 mM Ca^{2+} in the absence (a) or presence (b) of 4 μg/mL synexin. Ca^{2+} was added at time zero to initiate fusion of a 1-mL solution containing 10 μM phospholipid and the appropriate amount of synexin. Fusion was measured by the Tb/DPA assay as described in the text. Experiments were performed at 25 °C in NaCl buffer. No correction for leakage was necessary.

The second approach to changing the rate-limiting step involved the use of liposomes with different lipid compositions. To this end, we examined PA/PE/PC and PS/PE/PC (25:65:10) liposomes as opposed to PA/PE (25:75) and PS/PE (25:75) liposomes. The kinetics of fusion of these PC-containing liposomes are completely controlled by the fusion rate constant, as shown by a lack of dependence on the liposome concentration. The PA/PE/PC liposomes fused with no leakage of contents, and the overall fusion rate was independent of lipid concentration (10–20 μM) and Ca^{2+} concentration (3–5 mM) (not shown). The overall fusion rate for the PA/PE/PC liposomes in 5 mM Ca^{2+} was independent of the presence of synexin, at the level of 1 synexin per 120 phospholipids (data not shown), in contrast to PA/PE (25:75) liposomes (Figure 2). In Figure 4, it is shown that the fusion of PA/PS/PE/PC (10:15:65:10) liposomes, i.e., where some of the PA is replaced by PS, was dramatically inhibited by the presence of synexin at the same ratio. PS/PE/PC (25:65:10) liposomes aggregated, but did not fuse, in 5 mM Ca^{2+}.

In Table I, we have collected the qualitative data from this and previous studies on the effect of synexin on aggregation and fusion rates as a function of phospholipid composition and ionic conditions. In general, it appears from all the different liposome systems under study that synexin enhances liposome aggregation rates, whereas fusion rates are inhibited or remain unchanged. We will discuss the quantitative aspects and possible mechanisms for these processes below.

Table I: Effect of Synexin on the Ca^{2+}- and/or Mg^{2+}-Induced Fusion of Phospholipid Vesicles

phospholipid	aggregation rate ($\hat{C}_{11}$)	fusion rate ($\hat{f}_{11}$)	overall fusion rate	comment
PS	enhanced	no effect	enhanced or no effect[a]	up to 1 synexin/80 PS molecules (this paper)
PA/PE and PS/PE (1:3)	enhanced	nd[b]	enhanced	see Figure 2
PI/PE	nd[c]	nd[c]	inhibited	Hong et al. (1981)
PS/PC	enhanced	no fusion	no fusion	Hong et al. (1981)
PA/PE/PC (25:65:10)	nd[d]	no effect	no effect	$\hat{f}_{11}$ is completely rate limiting
PS/PE/PC (25:65:10)	nd[e]	no fusion	no fusion	up to 10 mM Ca^{2+}
PA/PS/PE/PC (10:15:65:10)	nd[d]	inhibited	inhibited	see Figure 4

[a] The overall rate of fusion was enhanced when the kinetics were largely aggregation rate limited (fusion induced by 3 mM Ca^{2+}), and no effect was observed when overall fusion was largely fusion rate limited (fusion induced by 4 mM Ca^{2+} and 5 mM Mg^{2+}). [b] The fusion rate constant could not be determined because the aggregation step was completely rate limiting. [c] Not determined. [d] The aggregation rate constant could not be determined because the fusion step was completely rate limiting. [e] No fusion was observed in this system.

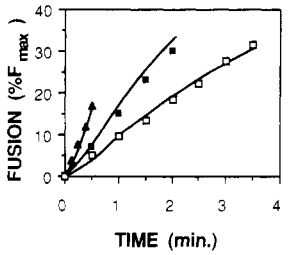

FIGURE 5: Modeling of the overall kinetics of fusion of PA/PE (1:3) liposomes in the presence of synexin. 2 mM Ca^{2+} was added at time zero to initiate fusion of liposomes in a cuvette containing (▲) 50 μM phospholipid and 11.8 μg/mL synexin, (■) 20 μM phospholipid and 4.7 μg/mL synexin, or (□) 10 μM phospholipid and 2.35 μg/mL synexin. The synexin:phospholipid ratio was kept constant. Fusion was measured by the Tb/DPA assay as described in the text. Experiments were performed at 25 °C in NaCl buffer. The solid lines represent the theoretical fusion curves calculated by the procedure described in the text. The rate constants obtained from these fits are shown in Table II.

Table II: Effect of Synexin on the Rate Constants for Aggregation and Fusion Initiated by 2 mM Ca^{2+} [a]

phospholipid	synexin/phospholipid	$\hat{C}_{11}$ (M^{-1} s^{-1})	$\hat{f}_{11}$ (s^{-1})
PS/PE (1:3)	0	$(5.0 \pm 0.5) \times 10^5$	$>5 \times 10^{-2}$
	1/400	$(1.0 \pm 0.3) \times 10^7$	$(3 \pm 1) \times 10^{-2}$
PA/PE (1:3)	0	$(5.5 \pm 0.5) \times 10^5$	$>10^{-1}$
	1/400	$(2.5 \pm 0.1) \times 10^6$	$(3 \pm 2) \times 10^{-2}$
	1/200	$(2.0 \pm 0.2) \times 10^7$	$(5 \pm 2) \times 10^{-2}$
PA/PS/PE/PC (10:15:65:10)	0	nd[b]	7×10^{-3}
	1/120	nd[b]	4×10^{-3}

[a] The aggregation $[\hat{C}_{11} = C_{11}/(1 + D_{11}/f_{11})]$ and fusion $[\hat{f}_{11} = f_{11}(1 + D_{11}/f_{11})]$ rate constants were fit by the procedures described in detail in Bentz et al. (1983a, 1985) and Düzgünes, and Bentz (1987). For the PA/PE vesicles, there was no leakage of contents on the time scale used. For the PS/PE vesicles, there was some leakage, and this leakage was used to correct the fusion signals to obtain the total amount of mixed contents according to the formula described in the text. [b] The aggregation rate could not be observed because the reaction was completely fusion rate limited.

We have quantitated the effect of synexin on aggregation and fusion rate constants ($\hat{C}_{11}$ and $\hat{f}_{11}$) by using fusion data at several concentrations of liposomes. Figure 5 shows the fusion of several concentrations of PA/PE (1:3) liposomes induced by 2 mM Ca^{2+} in the presence of synexin at a ratio of approximately 1 synexin molecule per 200 phospholipids. The Tb/DPA fusion signals are shown by the data points for 50, 20, and 10 μM total lipid. For these liposomes, there was no significant leakage of contents on the time scale shown. The data were simulated by employing the mass action kinetic model (eq 1) which has been used previously to fit rate constants to observed fluorescence data (Nir et al., 1980, 1983; Bentz et al., 1983a,b, 1985; Düzgüneş & Bentz, 1988). With a 1:1 ratio of Tb- to DPA-containing liposomes, it is not possible to rigorously fix all three primary rate constants in the time regime where higher order reactions are negligible (Bentz et al., 1983a; Düzgüneş & Bentz, 1988). However, we can obtain $\hat{C}_{11} = C_{11}/(1 + D_{11}/f_{11})$ and $\hat{f}_{11} = f_{11}(1 + D_{11}/f_{11})$. When aggregation is irreversible, then $\hat{C}_{11} = C_{11}$ and $\hat{f}_{11} = f_{11}$.

Using a wide range of lipid concentrations, we determined values of $\hat{C}_{11}$ and $\hat{f}_{11}$ for the fusion of PA/PE and PS/PE (1:3) liposomes induced by 2 mM Ca^{2+} with and without synexin. These data are shown in Table II. Without synexin, the PA/PE and PS/PE liposomes aggregated at about the same rate ($\hat{C}_{11} \sim 5 \times 10^5$ M^{-1} s^{-1} in both cases). These aggregation rate constants were sufficiently small that we could not measure the fusion rate constant ($\hat{f}_{11}$) without using excessively large lipid concentrations (>500 μM). Thus, we could only estimate the lower bounds for the fusion constants in these cases.

Adding synexin at the level of 1 synexin per 400 phospholipids sharply increased the aggregation rate constants by about 20-fold for the PS/PE liposomes and about 4–5-fold for the

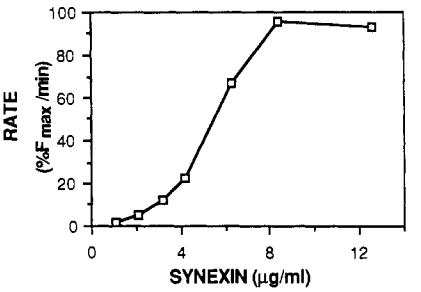

FIGURE 6: Rate of fusion of PA/PE (1:3) liposomes as a function of synexin concentration. 2 mM Ca^{2+} was added at time zero to initiate fusion of a 1-mL solution containing 10 μM PA/PE liposomes and the appropriate amount of synexin. Fusion was measured by the Tb/DPA assay as described in the text. All experiments were performed at 25 °C in NaCl buffer.

PA/PE liposomes under the conditions used. At other synexin:phospholipid ratios, the PA/PE liposomes aggregate as rapidly or more rapidly than PS/PE liposomes. Doubling the amount of synexin added to the PA/PE liposomes to 1 synexin per 200 phospholipids further enhanced $\hat{C}_{11}$ by 8-fold or a 36-fold increase over $\hat{C}_{11}$ in the absence of synexin. Although it is difficult to quantitate the effect of synexin on $\hat{f}_{11}$ in these cases, it would appear to have produced a slight inhibition (Table II).

The value of $\hat{f}_{11}$ could be directly measured for liposomes composed of PA/PS/PE/PC (10:15:65:10). Table II shows that the presence of 10% PC reduced $\hat{f}_{11}$ by over an order of magnitude to 7×10^{-3} s^{-1}, compared to the PA/PE and PS/PE liposomes. Under these conditions, synexin decreased $\hat{f}_{11}$ by about 2-fold at a ratio of 1 synexin per 120 phospholipids.

Synexin Interaction with Phospholipids. Some of the details of the nature of the interaction of synexin with phospholipid

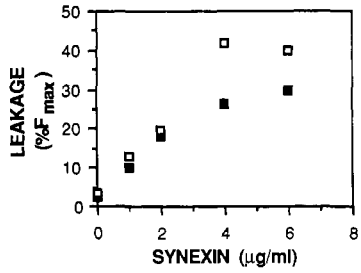

FIGURE 7: Leakage of contents from PS liposomes as a function of synexin concentration when fluorescence due to contents mixing has reached 10% maximal. Fusion of 10 μM PS liposomes with the appropriate amount of synexin was initiated by adding 3 mM Ca^{2+} (■) or 4 mM Ca^{2+} and 5 mM Mg^{2+} (□). The fusion of ANTS-containing and DPX-containing liposomes was measured, and the time at which the fluorescence reached 10% of maximal was noted. The amount of leakage at this time was then measured by following the time course of leakage of 10 μM PS liposomes containing both ANTS and DPX under the same conditions, as described in the text. The leakage, expressed in terms of a percentage of the maximal fluorescence which would be observed upon total leakage, is plotted against the synexin concentration.

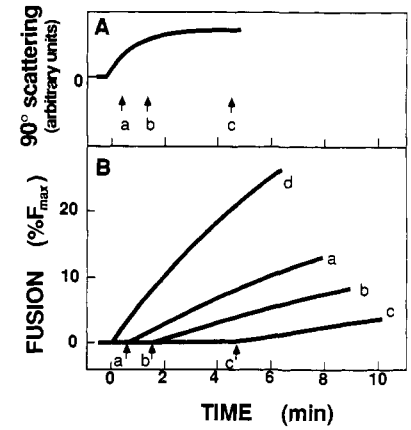

FIGURE 8: (A) Time course of the change in 90° light scattering at 276 nm induced by the addition of 1 mM Ca^{2+} at time zero to a solution of 2.6 μg/mL synexin. The arrows indicate 0.4 (a), 1.4 (b), or 4.6 (c) min. (B) The time course of the fusion of added PA/PE (1:3) liposomes as determined by the fluorescence at >530 nm of Tb^{3+} in the Tb/DPA assay. 90° light scattering was monitored as above after the addition of 1 mM Ca^{2+}. Then, 10 μM phospholipid was added at 0.4 (a), 1.4 (b), or 4.6 (c) min, and fusion was monitored in the other channel of the T-format fluorometer. In curve d, 1 mM Ca^{2+} was added to a solution already containing 10 μM phospholipid and 2.55 μg/mL synexin. All experiments were performed at 25 °C in NaCl buffer.

bilayers were revealed by investigation of the stoichiometry of interaction and the effect on the permeability of the bilayer. Figure 6 shows the initial rate of Tb/DPA fluorescence increase for 10 μM PA/PE (1:3) liposomes in the presence of 2 mM Ca^{2+} and various synexin concentrations. There was a sharp rise in the rate after approximately 2 μg/mL (or 1 synexin per 235 phospholipids), but the effect reached a plateau at ~8 μg/mL (or 1 synexin per 56 phospholipids). Adding more synexin (up to 13 μg/mL) did not change the overall fusion rates. In the Discussion, we interpret the saturation of the synexin effect in Figure 6 in terms of binding saturation.

Another characteristic indicator of the interaction of synexin with phospholipids is the extent to which synexin promotes leakage of contents during Ca^{2+}-induced fusion. With the PA/PE (1:3) liposomes, there was little leakage of contents with or without synexin at the early stages. For PS/PE (1:3), the leakage was significant and increased with increasing synexin concentration. However, the leakage per fused liposome was relatively low. By contrast, synexin promotes a large rate of leakage along with mixing of contents for pure PS liposomes. The important parameter here is how much leakage occurs per fusion event in the presence of various amounts of synexin. In Figure 7, the percent leakage of contents at the time when the fluorescence due to contents mixing reached 10% of maximum (i.e., about 20% of the aqueous contents have mixed and not leaked) is plotted against synexin concentration. In this way, we see how much leakage of contents occurs for a given extent of mixing of contents within fused liposomes. For example, in the presence of 2 μg/mL synexin, when the fluoresence due to contents mixing reaches 10% maximal, about 20% of the original contents of the liposomes is present in fused liposomes while about 19% of the original contents has leaked. Clearly, the leakage during fusion increases with synexin concentration, and, to some extent, it is larger when initiated by 4 mM Ca^{2+} and 5 mM Mg^{2+}, where fusion is the rate-limiting step.

Synexin Polymerization. We have also examined the role of synexin polymerization in liposomal aggregation and fusion. In Figure 8A, the kinetics of polymerization of synexin are shown using 90° light scattering. The change in scattering intensity was monitored as a function of time. When Ca^{2+} was added, the scattering intensity of the synexin solution increased. In Figure 8B, we show the effect of Ca^{2+}-induced synexin polymerization on the fusion of PA/PE (1:3) liposomes in 1 mM Ca^{2+}. Liposomes were added to the synexin solution at various stages of the Ca^{2+}-induced aggregation. It is clear that addition of liposomes after synexin polymerization produced an inhibition of the synexin effect on the overall rate of fusion. This inhibition may be due either to diminished activity in prepolymerized synexin or to a decrease in the number of particles of synexin.

In an attempt to determine whether polymers of synexin as well as monomers are active, we tested conditions where the approximate average number of synexin particles before fusion was known and held constant. To accomplish this, synexin aggregation was monitored in the absence of liposomes using 90° light scattering to obtain a fixed average polymer size. As described by Kerker (1969), for particles small compared with the wavelength of light (ca. 20 times smaller in their longest dimension), the relative increase of scattered light due to polymerization will be equal to the mass average polymer size. One may set the scattered light intensity of the buffer to zero and the intensity of the monomers to 1 to obtain a convenient scale for estimating the average polymer size, e.g., it will equal 2 if all of the particles dimerize. This procedure has been used to measure the aggregation of small sonicated liposomes (Lansman & Haynes, 1975; Bentz & Nir, 1981). Using this method, we found that the dimerization rate constant for synexin in 1 mM Ca^{2+} is about 5×10^5 M^{-1} s^{-1} (data not shown). The importance of this rate is discussed below.

The effect of synexin preaggregation on the overall aggregation and fusion of liposomes is shown in Figure 9. In particular, Figure 9c shows the case where the scattered light intensity increased 6-fold before the liposomes were added. Here we known that the average synexin polymer size is at least six monomers, and if the polymers are too large to follow Rayleigh–Debye scattering theory, then the average size is even larger (Kerker, 1969). Figure 9d shows that the same amount of synexin in monomeric form, i.e., without preaggregation, produces much faster overall fusion. Thus, six monomers are more active with respect to aggregation of liposomes than one "hexamer". If the preaggregated synexin is somewhat larger, then the difference would be even greater. On the other hand, each preaggregated synexin polymer promotes faster liposomal

aggregation than each monomer. This is based upon comparing hexamers (Figure 9c) with one-sixth the concentration of monomers (Figure 9b), so that the particle number is assumed to be the same in both cases (underlying assumptions are discussed below). Again, if the preaggregates are somewhat larger, then the difference between the two cases would be even greater. Similar results were obtained when synexin was preaggregated to only an average size of "trimers" (data not shown).

In contrast to the PA/PE liposomes, the apparent activity of polymers was not observed in a system largely limited by the fusion rate. The rate of fusion of PS liposomes induced by 4 mM Ca^{2+} and 5 mM Mg^{2+} (data not shown) was not increased significantly by either prepolymerized synexin or untreated synexin. Therefore, under these largely fusion rate-limited conditions, neither the polymerized synexin nor the untreated synexin is effective.

Discussion

Synexin Effects on Aggregation vs Fusion. Previous studies (Hong et al., 1981, 1982a,b) showed that, except for the case of PI/PE, synexin dramatically enhances the overall rate of fusion of acidic liposomes and reduces the threshold concentration of Ca^{2+} required for fusion. Those studies were consistent with previous studies showing promotion of aggregation of bovine chromaffin granules by synexin (Creutz et al., 1978; Morris & Hughes, 1979). If certain free fatty acids were added, synexin could also induce fusion of chromaffin granules (Creutz, 1981) or specific granules from human neutrophils (Meers et al., 1987a). By focusing separately on the process of aggregation versus the actual fusion, we were able to elucidate more mechanistic details about the effect of synexin on the Ca^{2+}-induced fusion of large unilamellar liposomes.

Table II indicates that the rate constants of aggregation are significantly increased by the addition of synexin to either PA/PE or PS/PE liposomes. The cases analyzed in Table II correspond to the aggregation rate-limiting case, $\hat{f}_{11} \gg \hat{C}_{11}X_0$, where X_0 is the molar concentration of liposomes. Within the uncertainty of these calculations, we can deduce that synexin significantly increases the aggregation rate, but we cannot determine the effect on the fusion rate constants. In order to supplement this information, experiments were designed to investigate the effect of synexin in cases where the actual fusion step is rate limiting to the overall process. In this extreme case, the changes in liposome concentration should not affect the overall fusion rate. This was indeed the case for PA/PS/PE/PC (10:15:65:10) liposomes and PA/PE/PC (25:65:10) liposomes, where PC helps to render these systems fusion rate limited. Figure 6 and Table II demonstrate that the addition of synexin (1/120 synexin per phospholipid) results in a slowing of fusion kinetics of PA/PS/PE/PC; i.e., the fusion rate constant decreases by a factor of 2 due to bound synexin. For PA/PE/PC (25:65:10), there was no change of the fusion rate constant. This agrees with the lack of effect of synexin observed by Hong et al. (1982b) for PA/PE/PC (25:50:25) liposomes.

In a related experiment, we utilized the fact that Mg^{2+} promotes the aggregation of PS liposomes without inducing their fusion or leakage (Wilschut et al., 1981; Bentz & Düzgüneş, 1985). Thus, Mg^{2+} was used to render the PS system nearly fusion rate limited. When 4 mM Ca^{2+} and 5 mM Mg^{2+} were used to induce fusion, the overall fusion rates were practically unaffected by synexin (Figure 5). Without Mg^{2+}, where aggregation was rate limiting, the addition of synexin enhanced the overall fusion kinetics. This primarily aggregating effect of synexin is consistent with previous data on the increased Ca^{2+}-induced aggregation of PS/PC (1:3) liposomes monitored by turbidity changes (Hong et al., 1981). While there was Ca^{2+}-dependent aggregation, no fusion of the PS/PC liposomes was observed either with or without synexin. In summary, our results show that promotion of the overall rate of fusion occurred when the aggregation step was rate limiting and no effect, or inhibition, was observed when the actual fusion step was rate limiting (Table I). Therefore, it is clear that, under the varied conditions tested, synexin only increases the rate of aggregation of liposomes but not the rate of actual fusion.

In contrast to these results, Ohki and Leonards (1982) found that synexin decreases the initial rate of Ca^{2+}-induced aggregation of sonicated PS liposomes, yet increases the final extent of aggregation. On the basis of reversibility of turbidity changes, it was concluded that synexin does not increase the final extent of fusion of liposomes, but it is not clear if irreversible aggregation could have contributed to this result. The reasons for the discrepancy of this study with the results of other investigators are not known but may have to do with the fact that smaller sonicated liposomes were used and the synexin to lipid ratio was very low.

Similar results to ours have been very recently reported by Nir et al. (1987), showing that synexin promotes the H^+-dependent (Ca^{2+}-independent) fusion of chromaffin granules that have been frozen and thawed. This pH-dependent fusion was promoted mainly by increasing the rate of granule aggregation. The fusion rate constant was independent of the synexin concentration except at very high synexin to lipid ratios, where the rate constant was significantly reduced, and at very low ratios, where it was slightly enhanced.

The phenomenon of promotion of Ca^{2+}-induced fusion of liposomes was also reported by Düzgüneş et al. (1984), who showed that lectins can increase the rate of fusion of glycolipid-containing liposomes. In this case, lectins may also be acting like synexin in that they may enhance the rate of aggregation of liposomes. Similar results were also reported by Gad et al. (1985), who employed cardiolipin/phosphatidylcholine liposomes and the positively charged polypeptide polylysine or lysine-based copolymers (Gad et al., 1986). Ca^{2+} was required for fusion, but the overall fusion reaction was promoted at certain polylysine concentrations, and the Ca^{2+} threshold concentration for fusion could be reduced. However, excess polylysine caused a reduction in the fusion rate constant, even when it did not result in a reduction in the rate of liposomal aggregation (Gad et al., 1985). Although synexin is not a highly positively charged protein (Creutz et al., 1983) and requires relatively low Ca^{2+} concentrations for activity (Hong et al., 1982b), these effects on the fusion rate constant are somewhat analogous to what we observed in the case of PA/PS/PE/PC liposomes with synexin.

In those cases where synexin actually inhibits fusion, it is possible either that the bound synexin physically blocks the close approach of the lipid bilayers or that it reduces the number of free lipids available at the sites of close apposition where fusion can occur. In the case of PA/PS/PE/PC liposomes, this latter alternative would require that synexin binding causes the apposed bilayer area to be depleted of PA since the PS/PE/PC liposomes do not fuse. On the other hand, the PS may just bind more synexin which then physically blocks close apposition of the bilayers.

It may be that in the synexin-aggregated liposome system, fusion occurs at a site away from where synexin is bound. While this is merely speculation at this point, several other observations also support this suggestion. First, synexin only

decreases a finite threshold for Ca^{2+}-induced fusion. Therefore, the ability of the lipids to fuse at some Ca^{2+} concentration, without synexin, is required. Second, PC does not bind to synexin and is inhibitory to liposome fusion in the absence and presence of synexin (Tables I and II; Hong et al., 1982b). Since we assume that the presence of PC does not affect synexin binding in any way (when the phospholipid is in excess), we must conclude that the effect of PC on fusion is exerted at some site away from where synexin is binding. Though we have little information about the structure of the liposome–synexin aggregate, it is not unreasonable to expect that a relatively bulky protein molecule between apposed bilayers at the site of fusion could inhibit fusion of the bilayers, while aggregation of liposomes could still occur.

In light of our analysis, we can describe in more detail the result of Hong et al. (1982a), who showed that addition of as little as 10–30 μM Ca^{2+} could produce fusion of PA/PE (1:3) liposomes in the presence of synexin and 1.5 mM Mg^{2+}. Both Ca^{2+} and Mg^{2+}, at higher concentrations, will cause these vesicles to fuse. Thus, it is most likely that the small Ca^{2+} concentrations activated the synexin to aggregate the liposomes, and the membrane-bound Mg^{2+} induced the fusion; 1.5 mM Mg^{2+} without synexin could not promote the aggregation step. Without the Mg^{2+}, the synexin and Ca^{2+} induced no fusion, probably because electrostatic repulsion between the vesicles was too large without further charge neutralization.

Synexin Interaction with Phospholipids. While the data presented here do not provide detailed information on the binding of synexin to bilayers, the following observations, in addition to the information obtained from fusion kinetics, are noteworthy. The data in Figure 6 show that the initial rate of fluorescence increase for PA/PE (1:3) liposomes rose with synexin concentration, reaching a plateau at 1 synexin molecule per 56 phospholipid molecules. We note that if all the synexin is bound to the liposomes [as in Meers et al. (1987b)], and if synexin is taken as a cylinder 30 Å in diameter and 80 Å long, then close packing of synexin (end-on) on the liposomal surface would yield about 1 synexin per 10 phospholipids on the outer bilayer surface, using 70 $Å^2$/phospholipid. However, it is unlikely that random binding of synexin to the vesicles would permit the closest possible packing, and it is possible that not every synexin molecule makes contact with the membrane. Nonetheless, the plateau in overall fusion rates may simply demark the lack of additional binding sites for synexin.

The leakage of contents per synexin molecule may also be relevant to the binding to phospholipids. The largest amount of leakage per fusion event is observed in pure PS liposomes as opposed to PS/PE and PA/PE liposomes. We do not know if this is due to synexin penetration into the PS liposomes, making them more leaky, or a more indirect mechanism of destabilization. It is known that synexin contains hydrophobic sequences which could, in principle, penetrate into the bilayer (Pollard et al., 1987), but it has not yet been established that synexin actually penetrates into liposomal bilayers. PS/PC (1:1) liposomes are not made leaky by synexin binding and synexin-mediated aggregation (Hong et al., 1981) in the absence of fusion, nor are PA or PS liposomes made leaky by synexin binding alone in the absence of aggregation and fusion (Meers et al., 1987c). Therefore, the differences in leakage observed between the various lipids in the presence of synexin may not be related to synexin binding but may be strictly associated with fusion of the liposomes and eventual collapse of larger fusion products.

Synexin Polymerization. Another question related to the action of synexin, is the role of Ca^{2+}-induced polymerization of synexin monomers. In contrast to the case of polyamines (Hong et al., 1983b; Schuber et al., 1983; Meers et al., 1986) or polylysine (Gad et al., 1985) which can aggregate acidic liposomes without Ca^{2+} (or other cations), the activity of synexin requires the presence of Ca^{2+}. For instance, Mg^{2+} cannot act as a substitute. Furthermore, it has been shown that synexin is polymerized by Ca^{2+}, but not by Mg^{2+} or Sr^{2+}, and only slightly by Ba^{2+} (Creutz et al., 1979). While we favor the idea (Creutz et al., 1978, 1979; Pollard et al., 1987) that the molecular changes in synexin that are induced by Ca^{2+} are specific and essential for its action, there is no evidence that polymerization of synexin is necessary for its action.

Monitoring the 90° light scattering due to synexin polymerization with Ca^{2+}, we found that the rate constant of synexin polymerization is about 5×10^5 M^{-1} s^{-1}. This value is consistent with the synexin polymerization data presented by Creutz et al. (1979). This constant is about 2 orders of magnitude lower than the liposome aggregation rate constants in the presence of synexin and Ca^{2+}. It would appear that the kinetically favored pathway is synexin binding to liposomes before self-aggregation could occur. On the other hand, once bound to one membrane surface, there is no reason why liposome aggregation could not include dimers (Creutz et al., 1987) or larger polymers of synexin.

The results in Figure 8A,B demonstrate that polymerization of synexin, in the absence of a phospholipid membrane, does not result in enhancement of the initial overall fusion rates. On the contrary, preincubation of synexin in the presence of Ca^{2+}, prior to the addition of liposomes resulted in reduced initial fusion rates, reflecting either the reduction in the number of total synexin units or a reduction in the activity of the polymerized synexin. Our results in Figure 9 show that polymerized synexin has less total activity than monomers, and may imply that a polymer (up to hexamers) is more active than a single monomer.[2] One can speculate that one reason for the greater than expected activity of prepolymerized synexin under strongly aggregation rate-limiting conditions is that two or more synexin molecules can act as a longer cross-linker between aggregated bilayers, overcoming some of the repulsive forces between negatively charged liposomes simply by distance. It is not known how this kind of geometry would affect the fusion rate, especially if fusion occurs at a site away from where synexin binds to the liposome. In contrast to the effect of prepolymerization observed when aggregation was rate

[2] If synexin polymerizes like most known protein polymers, with a continuous unimodal distribution (Oosawa & Asakura, 1975; Goldstein & Stryer, 1986), then we know that the hexamer is more active than is the monomer. Under these conditions, by the time that the average polymer size is six, the concentration of monomers is negligible (Bentz & Nir, 1981). Thus, the observed activity of the preaggregated synexin cannot be due to a residual pool of monomers. Our concern is that an average polymer size of six could be composed of monomers and a few giant, but inactive, polymers, due to some sort of nucleation process. Here it would be possible to construct a case where preaggregation of synexin depletes the monomer pool below 2.5 μg/mL (as in Figure 9d) but leaves it above 0.41 μg/mL (as in Figure 9b) at the time of the 6-fold increase in scattered light intensity. It is unlikely that synexin follows any sort of nucleation process of monomers to giant polymers, since Creutz et al. (1979) report that the initial rate of aggregation is a second-order process with respect to synexin concentration. The complete answer must await a detailed kinetic analysis of the size distribution. It is also possible that liposomal membranes might cause some dissociation of preformed polymers of synexin, i.e., if the affinity of synexin for the membrane is greater than for other synexin molecules. Thus, the number of monomers may be greater than expected, also explaining the greater than expected activity.

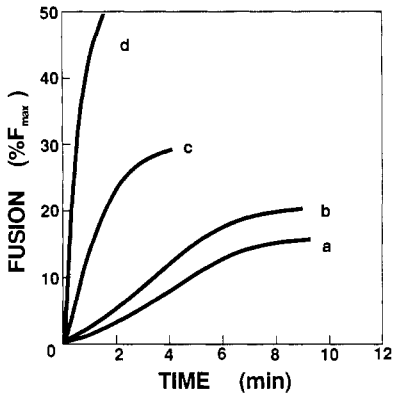

FIGURE 9: Effect of prepolymerization of synexin on the fusion of PA/PE (25:75) liposomes: (a) 2 mM Ca^{2+} was added at time zero to 10 μM PA/PE liposomes; (b) 2 mM Ca^{2+} was added at time zero to 10 μM PA/PE liposomes and 0.41 μg/mL synexin; (c) 2 mM Ca^{2+} was added to 2.5 μg/mL synexin, the synexin was allowed to polymerize to an average aggregate size of 6 as determined by 90° light scattering (see Materials and Methods), and then 10 μM PA/PE liposomes were added at zero time; (d) 2 mM Ca^{2+} was added to 10 μM PA/PE liposomes and 2.5 μg/mL synexin. All experiments were performed at 25 °C in NaCl buffer.

limiting, prepolymerization did not increase synexin activity under fusion rate-limiting kinetics with the pure PS liposomes. This is consistent with the action of synexin only at the aggregation step in the kinetics of liposome fusion.

Biological Relevance. Fusion of intracellular membranes would appear to require at least three steps: (1) specific recognition of the site of fusion; (2) close apposition of membranes; (3) fusion or coalescence of membranes. Whether synexin could control the rate of secretion or any other intracellular membrane fusion event depends on which of the above steps is rate limiting. Our results, along with those of others, suggest that if synexin is involved in intracellular membrane fusion it acts at step 2 by bringing together membranes in a Ca^{2+}-dependent manner, so that membrane fusion events, such as secretion, can occur. Another factor besides synexin or some modification of the synexin molecule, such as phosphorylation or enzymatic production of free fatty acids, diacylglycerols, or phosphatidate by phospholipases, would probably be needed in modulating the actual fusion of membranes. Nonetheless, synexin's possible role in these processes continues to be intriguing.

Registry No. Ca, 7440-70-2.

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A Series of Fluorescent *N*-Acylsphingosines: Synthesis, Physical Properties, and Studies in Cultured Cells†

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ABSTRACT: We have previously shown that when cultured fibroblasts are briefly incubated at 2 °C with a fluorescent (NBD) analogue of ceramide, *N*-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ε-aminohexanoyl]-D-*erythro*-sphingosine, fluorescent labeling of the mitochondria, endoplasmic reticulum, and nuclear envelope occurs. During further incubation at 37 °C, the Golgi apparatus and later the plasma membrane become intensely fluorescent. Concomitantly, the fluorescent ceramide is metabolized to fluorescent analogues of sphingomyelin and glucosylceramide [Lipsky, N. G., & Pagano, R. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2608–2612]. In the present study we synthesized fluorescent *N*-acylsphingosine analogues using various long-chain bases (D-*erythro*-sphingosine, L-*erythro*-sphingosine, D-*threo*-sphingosine, L-*threo*-sphingosine, D-*erythro*-dihydrosphingosine, L-*threo*-dihydrosphingosine, phytosphingosine, and 3-ketosphingosine) and fluorescent fatty acids (ε-NBD-aminohexanoic acid; D- or L-α-OH-ε-NBD-aminohexanoic acid; D- or L-α-NBD-aminohexanoic acid). Using previously described resonance energy transfer assays, we examined the rates of spontaneous transfer of these compounds between liposomes and their ability to undergo transbilayer movement. The fluorescent *N*-acylsphingosine analogues had half-times for spontaneous transfer of 0.3–4.0 min at 25 °C, and all were capable of transbilayer movement in lipid vesicles. The metabolism and intracellular distribution of analogues in cultured fibroblasts were also studied. While most of the fluorescent *N*-acylsphingosines were significantly metabolized to the corresponding sphingomyelin analogues, metabolism to glucosylceramide was strongly dependent on the long-chain base and the stereochemistry of the fluorescent fatty acid moiety. When cells were incubated with the various *N*-acylsphingosine analogues under appropriate conditions, labeling of the Golgi apparatus was seen in all cases except for *N*-(ε-NBD-aminohexanoyl)-3-ketosphingosine.

We have developed a series of fluorescent (NBD)[1] lipid derivatives which are useful in studying the synthesis, molecular sorting, and intracellular transport of lipids in animal cells [reviewed in Pagano and Sleight (1985)]. The metabolism of these lipids in cells can be studied by conventional lipid biochemical procedures, and these data can then be correlated with the intracellular distribution of these molecules and their metabolites within *living* cells by fluorescence microscopy. One lipid, a fluorescent analogue of ceramide, is particularly interesting because it prominently labels the Golgi apparatus of cells (Lipsky & Pagano, 1983, 1985a,b) and provides a means for studying the traffic of sphingolipids through this organelle (Lipsky & Pagano, 1983, 1985a; van Meer et al., 1987). When cells are treated with this lipid at low temperature, washed, and warmed to 37 °C, the Golgi apparatus and later the plasma membrane become intensely fluorescent. During this redistribution of intracellular fluorescence, the fluorescent ceramide is metabolized to fluorescent SM and GlcCer. Consistent with the increasing fluorescence at the plasma membrane over time, increasing amounts of each fluorescent metabolite can be removed from the cell surface by backexchange to nonfluorescent acceptor liposomes. Furthermore, the ionophore monensin, which inhibits the transport of newly synthesized glycoproteins to the cell surface, also inhibits the delivery of these fluorescent lipid metabolites to the cell surface and causes accumulation of fluorescence in the region of the Golgi apparatus. These results suggest that both the fluorescent SM and GlcCer analogues are synthesized intracellularly from the fluorescent ceramide precursor and then translocated through the Golgi apparatus

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[1] Abbreviations: BSA, bovine serum albumin; C, chloroform; DOPC, dioleoylphosphatidylcholine; GlcCer, glucosylceramide; HCMF, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Puck's saline without calcium or magnesium; HMEM, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered Eagle's minimal essential medium, pH 7.4, without indicator; HPLC, high-pressure liquid chromatography; M, methanol; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NHS, *N*-hydroxysuccinimidyl; RET, resonance energy transfer; SM, sphingomyelin; TLC, thin-layer chromatography.

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